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Research article

# REM sleep loss associated changes in orexin-A levels in discrete brain areas in rats

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## HIGHLIGHTS

- REMS loss differentially increases Orx-A levels in discrete brain regions.
- Maximum increase in Orx-A levels was observed in the LC, the site of REM-OFF neurons.
- Orx-A levels remained unaffected in the PPT, the site of REM-ON neurons.
- REMSD activates and REMS rebound inhibits Orx-ergic system.

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## ABSTRACT

Rapid eye movement sleep (REMS) serves house-keeping function of the brain and its loss affects several pathophysiological processes. Relative levels of neurotransmitters including orexin A (Orx-A) in various parts of the brain in health and diseases are among the key factors for modulation of behaviors, including REMS. The level of neurotransmitter in an area in the brain directly depends on number of projecting neurons and their firing rates. The locus coeruleus (LC), the site of REM-OFF neurons, receives densest, while the pedunculo-pontine area (PPT), the site of REM-ON neurons receives lesser projections from the Orx-ergic neurons. Further, the Orx-ergic neurons are active during waking and silent during REMS and NREMS. Therefore, the level of Orx-A in discrete regions of the brain is likely to be different during normal and altered states, which in turn is likely to be responsible for altered behaviors in health and diseases, including in relation to REMS. Therefore, in the present study, we estimated Orx-A level in LC, cortex, posterior hypothalamus (PH), hippocampus, and PPT after 96 h REMSD, in post-deprivation recovered rats and in control rats. This is the first report of estimation of Orx-A in different brain regions after prolonged REMSD. It was observed that after REMSD the Orx-A level increased significantly in LC, cortex and PH which returned to normal level after recovery; however, the level did not change in the hippocampus and PPT. The Orx-A induced modulation of REMS could be secondary to increased waking.



## 1. Introduction

Rapid eye movement sleep (REMS) is a unique reversible, physiological state expressed for varying duration; it repeats within the sleep state especially in the animals higher in evolutionary ladder. Normally it appears only after a period of non-REMS (NREMS) and barring in some diseased states (e.g. narcolepsy), REMS-like state does not appear during waking. Therefore, it was proposed that normally neurons responsible for REMS must be inhibited by the waking areas, while excited by the NREMS generating areas, which has been confirmed [1,2] and subsequently the complex neural connections for the regulation of REMS has recently been constructed [3]. It essentially suggests that as long as noradrenergic (NA)-ergic REM-OFF neurons in the locus coeruleus (LC) remain active, which of course are under the influence of varieties of neurotransmitter inputs, REMS is not expressed and those neurons must cease activity for the expression of REMS [3,4].

The levels of various neurotransmitters in different parts of the brain during REMS and REMS loss are likely to play significant role in REMS regulation during health and diseases. Orexin (Orx) is one of the many neurotransmitters involved in REMS regulation in health and diseases [5]. Orx synthesizing neurons are

*Abbreviations:* CSF, cerebrospinal fluid; ELISA, enzyme linked immunosorbent assay; FMC, free moving control; LC, locus coeruleus; PPT, pedunclo pontine tegmentum; LPC, large platform control; NREMS, non REMS; Orx-A, orexin-A; PeF, perifornical area; PH, posterior hypothalamus; REMS, rapid eye movement sleep; REMSD, REMS deprivation; REC, recovery.

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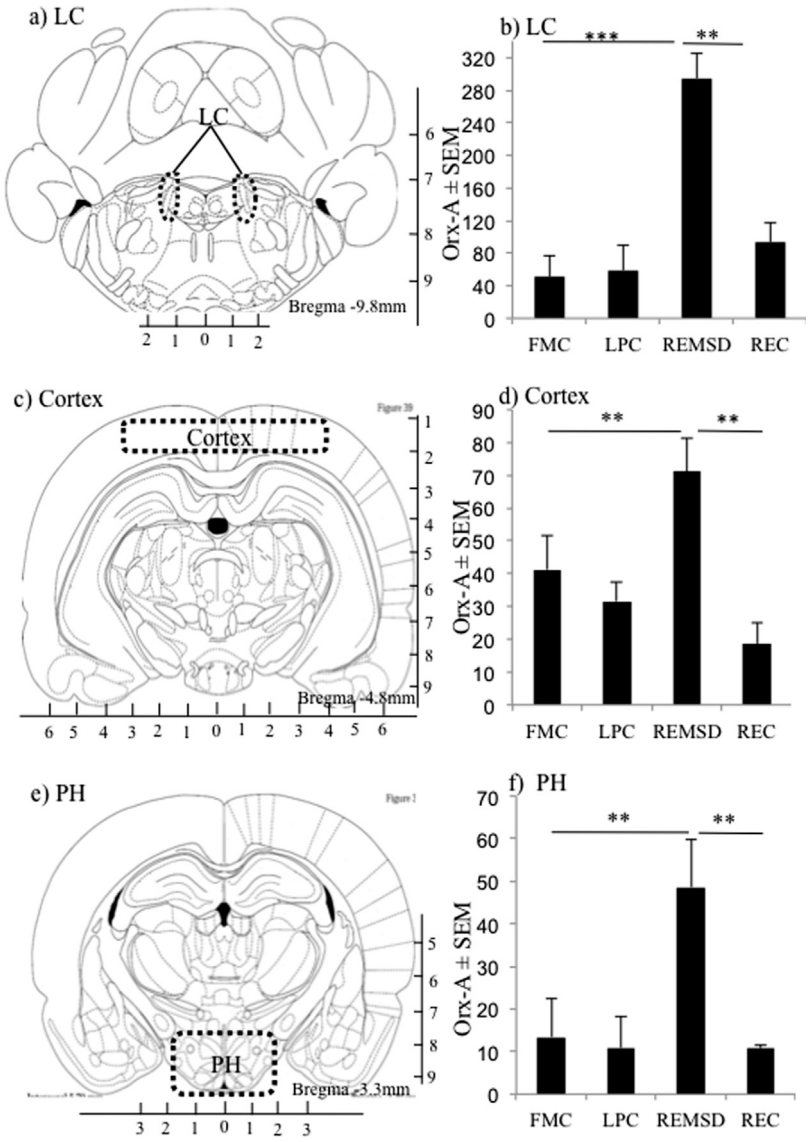


**Fig. 1.** Left panel shows coronal sections from the rat brain atlas [22]. The areas dissected out for estimation of Orx-A have been marked on the sections. The mean (±SEM) Orx-A level in the dissected portion ($n = 5$) has been shown in the corresponding histogram on the right panel. The Orx-A level increased in all the areas shown. Orx-A concentration is pg/g wet tissue weight. $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. Abbreviations are as in the text.

present in the perifornical (PeF) area, located at the lateral part of posterior hypothalamus (PH) and are generally active during waking, slow down and finally become quiescent during NREMS and REMS [6–8]. Activation of these neurons indeed increased waking and reduced REMS [9–11]. Recently, we have shown that Orx-ergic neurons indeed affect REMS by modulating the neurons in the LC [11]. Orx levels in the brain correspond to the activity of Orx-ergic neurons, i.e. higher during waking and REMS [12] and lower during NREMS. Interestingly, these Orx-ergic neurons project to many brain areas including the brainstem, LC, PH, which are classically known to be involved in the regulation of wakefulness-NREMS-REMS [11,13–15] and to hippocampus and cortex, among many other areas which are apparently not involved in sleep-waking-REMS regulation [16]. Also, Orx-ergic neurons in PeF have local self-collateral inputs [17].

All the neurons even in any one functional area in the brain do not behave and perform identically. Thus, it is obvious that the levels of the neurotransmitters in different projected regions of the brain are likely to be different during alteration in one function. The levels of neurotransmitters would contribute to pre-disposition of expressions of behavior and variations in quality and quantity of expressions of behavior including diseases and symptoms thereof. Therefore, it is essential to estimate the levels of a neurotransmitter in relation to changes in a particular function simultaneously in multiple areas of the brain where the neurons from one area project to. Thus, to address this issue, in the present study, we estimated Orx-A levels in control, after REMS deprivation (REMSD) and after recovery from REMSD in different brain areas known to be involved in REMS regulation and in the areas although not involved in regulation of REMS as such, but are known to modulate function(s) affected by REMS loss. As Orx is of two types Orx-A and Orx-B, in this study, we estimated levels of Orx-A. To the best of our knowledge, this is the first such study especially after long-term REMSD.

## 2. Materials and methods

### 2.1. Animals and REMSD

Experiments were conducted on inbred male Wistar rats (200–250 g; $n = 20$), maintained in 12:12 light/dark (L/D) cycle and

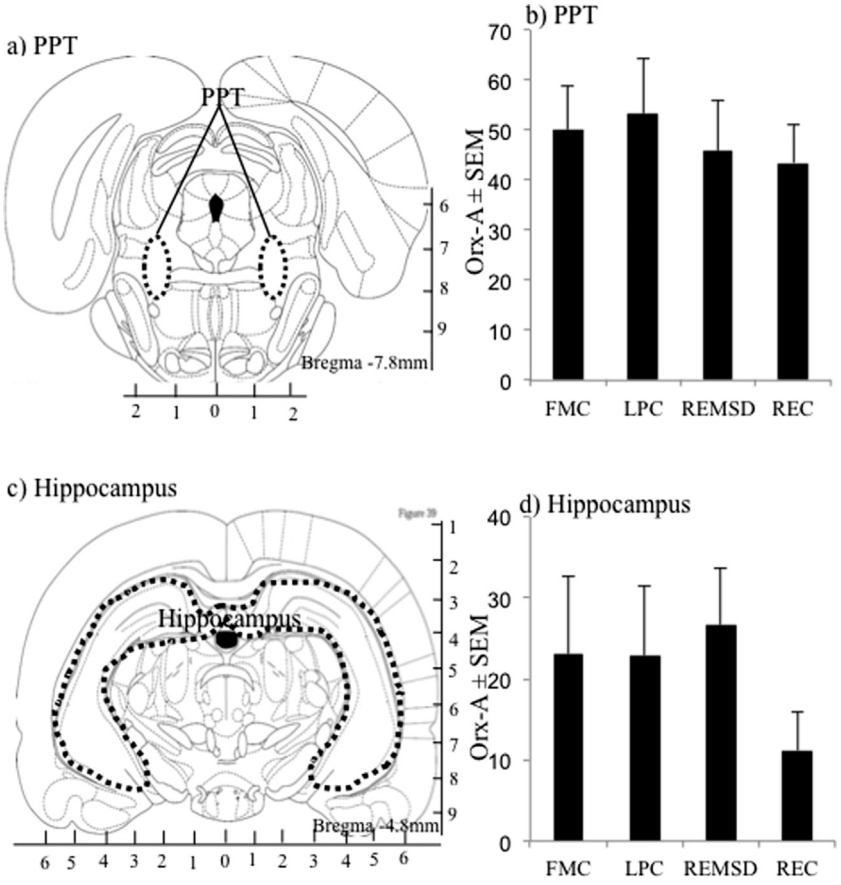


**Fig. 2.** Left panel shows coronal sections from the rat brain atlas [22]. The areas dissected out for estimation of Orx-A have been marked on the sections. The mean (±SEM) Orx-A level in the dissected area ($n = 5$) has been shown in the corresponding histogram on the right panel. The Orx-A level did not change significantly in any area shown. Orx-A concentration is pg/g wet tissue weight. Abbreviations are as in the text.

were supplied with food and water ad libitum. All the efforts were made to minimize the number of rats and their suffering. Free moving control (FMC) rats ($n = 5$) were maintained in their normal dry home cages. Experimental rats ($n = 5$) were REMS deprived by the classical flowerpot method as reported earlier [18,19]. In brief, for REMSD, the experimental rats were maintained for 4 days on a 6.5 cm diameter platform surrounded by water. In order to rule out the non-specific effects another group of rats ($n = 5$) was maintained on a larger (13 cm) platform (LPC) surrounded by water i.e., all the other conditions remained identical to the experimental animals except the size of the platform [19]. The fourth REC group included those animals ($n = 5$) which have been REMS deprived for 4 days and then kept in normal cages for 3 days to recover from REMS loss. Post-REMSD 3 days recovery were allowed as it was observed that this period was sufficient for the REMSD-induced changes in cellular morphology [20] and altered enzyme activities [19,21] to return to the level of FMC. Thus, each set of experiments had four rats, and five such sets were conducted, and the results are being reported here.

### 2.2. Tissue preparation

Rats were decapitated after cervical dislocation, and the brains were removed in chilled saline solution (0.9%). Brains were then sliced using a 1 mm slicer (World Precision Instruments, Inc., FL, USA) following marked areas (Figs. 1 and 2) as represented in sections of the rat brain atlas [22]. The bilateral LC (site of REM-OFF neurons) (Fig. 1a), cortex (Fig. 1c), PH (Fig. 1e), pedunculo-pontine tegmentum (PPT, site of REM-ON neurons) (Fig. 2a), and hippocampus (Fig. 2c) (the areas unrelated to REMS regulation but functions modulated by these brain areas are affected by REMS loss e.g., cognition, memory and thermoregulation) were dissected out. The tissues were weighed and homogenized in 0.1 M PBS at pH 7.4 on ice and then centrifuged at 10,000 × *g* for 10 min at 4 °C (Eppendorf centrifuge, Hamburg, Germany). Cortex, hippocampus and PH (relatively larger tissue samples) were homogenized to a concentration of 500 μg/μl by adding required volume of PBS. However, due to the smaller tissue sample LC and PPT were homogenized to 100 μg/μl. The supernatant was aliquoted and stored at −20 °C till further process.

### 2.3. Enzyme linked immunosorbent assay (ELISA) quantification of Orx-A

Stored samples were used for estimation of Orx-A using standard ELISA kit following the protocol supplied with the kit (Chongqing Biospes Co. Ltd., China). Briefly, equal volumes (50 μl) of homogenized samples were added to the 96 well plate and incubated at 37 °C for 45 min. The sample volumes were maintained constant for controls (FMC, LPC, REC) and experimental (REMSD) samples for each brain area. After incubation, the wells were washed with 1 × wash buffer and 50 μl of HRP-conjugated anti-Orx-A antibody was added and incubated at 37 °C for 40 min. Incubation was followed by washing and chromogenic reagent (tetra methyl benzidine) was added and kept for 15 min. The absorbance was recorded at 450 nm using an ELISA reader (Biotek Power Wave XS, USA). Standard curve for estimation of Orx-A was generated and the optical densities of the test samples were read

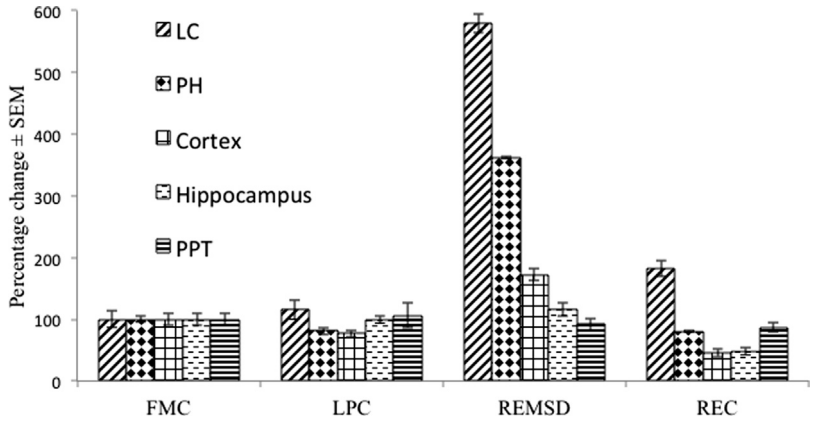


**Fig. 3.** Relative change in Orx-A level in different brain regions under varying conditions as compared to FMC (taken as 100) is shown here. The REMSD associated increased Orx-A returned to normal (FMC) level after REC. Abbreviations are as in the text.

against the standard curve. An average of duplicate readings of samples was calculated. The Orx-A has been represented as pg/g (pico-gram per gram) wet tissue weight.

### 2.4. Data analysis

Sigma Stat Statistical Software version 12 was used to analyze the data. One-way ANOVA with Student–Newman–Keuls post-hoc test was applied to evaluate the level of statistical significance of the differences in the levels of Orx-A in different regions of the brain after REMSD as compared to various controls. Each value was expressed as mean ± S.E.M., and statistical significance was considered at least at $p \leq 0.05$.

## 3. Results

The standard plot of absorption at 450 nm by various concentration of Orx-A showed a linear relationship with $R^2 = 0.993$. The wet tissue weight (mg ± SD) of each brain area across all rats taken for estimation of Orx-A was LC (24 ± 0.018), cortex (260 ± 0.113), PH (43 ± 0.022), PPT (25 ± .003) and hippocampus (126 ± 0.056). Under normal FMC condition detectable level (10–50 pg/g wet tissue) of Orx-A was estimated in samples from across the brain regions studied. The Orx-A levels were comparable in the FMC and LPC. In relative terms, in FMC rats the Orx-A levels in the cortex, PPT, LC were comparable; however, the level was relatively lower in the hippocampus and the PH (Figs. 1 and 2).

After REMSD the Orx-A levels increased significantly in LC ($F = 15.93$, $p \leq 0.001$, Fig. 1b), cortex ($F = 5.56$; $p \leq 0.01$, Fig. 1d) and PH ($F = 6.75$; $p \leq 0.01$, Fig. 1f). Further, increased levels of Orx-A returned (or tended to return) to the FMC level in the REC group where the animals got a chance to recover from REMSD (Figs. 1 and 2). However, the levels were not affected significantly in the PPT ($F = 0.036$; $p \leq 0.99$, Fig. 2b) and the hippocampus ($F = 0.556$; $p \leq 0.652$, Fig. 2d). The percent change in Orx-A level in different brain areas in REMSD, LPC and REC samples as compared to respective FMC is shown in Fig. 3. The maximum increase (fold change as compared to FMC) was seen in the LC, followed by in the PH and the cortex; hippocampus and PPT remained unaffected (Fig. 3). Interestingly, none of the areas showed a decrease in the levels of Orx-A after the REMSD.

## 4. Discussion

The Orx-A level could be detected in different brain regions, which may be explained by the fact that orx-ergic PeF neurons are known to project to those brain areas [23]. The difference in relative levels of Orx-A could be due to the density of projections and intensity of firing rate of the Orx-ergic projecting neurons from the PeF. Orx-ergic neurons widely innervate different brain regions and send their densest projections to the LC [24], which possesses REM-OFF and wake-active neurons [25]. During REMSD as the animal remains awake and the EEG continues to be desynchronized, the increased level of Orx-A in the LC could explain the role of Orx-A in arousal [26]. This may be supported by the fact that Orx-ergic neurons increase firing during waking and reduce firing during NREMS and REMS [6–8,27]. Our finding supports earlier observations that significantly elevated level of Orx-A has been reported during active-awake state as compared to quiet-awake animals [12], while its level was reduced after rebound sleep [28]. Increased level of Orx-A in LC during REMSD, supports the former's role in promoting waking and reducing REMS, possibly by increasing the activity of the LC mono-aminergic system in the brain [11]. The finding is consistent with our recent findings that activation of the PeF Orx-ergic neurons reduce REMS and increase waking by acting on the LC neurons [11]. Further, at the molecular level our results support previous findings that Orx receptor1 knock down in the LC facilitated REMS, while its rescue in narcoleptic mice restored sleep [29,30].

After REMSD the level of Orx-A increased in the cortex as well as in the PH. As discussed above the reasons for the increased level of Orx-A in these areas hold true as well because Orx-ergic neurons have been reported to project to these areas [16]. In the cortex the elevated level of Orx-A may stimulate the cortical neurons to induce EEG desynchronization, a characteristic feature of waking. The increased level of Orx-A in the PH after REMSD may be supported by the fact that PeF neurons in the PH are reported to have local self-collateral inputs [17] and those neurons increase firing during behavioral arousal [6,27,31]. As Orx has been reported to stimulate the glutamatergic neurons in the PH, a synergistic interaction between Orx-ergic glutamatergic neurons has been proposed to maintain arousal [32]. Also, Orx activated glutamate from the subcortical areas may contribute to maintain wakefulness [33]. Further, as the PH is known to be responsible for maintenance of homeostasis [13], we propose that normally the Orx-A may participate in maintaining sleep-waking homeostasis, which is disturbed upon REMSD resulting in elevated level of Orx-A or vice versa.

After REMSD the Orx-A level did not change in the PPT, the site of REM-ON neurons and the hippocampus, the site known to be involved in learning and memory. Therefore, it appears that Orx-A as such may not be directly regulating REMS and REMS related memory processes. As Orx-A increases waking and reduces NREMS, the effect on REMS could be secondary to reduced NREMS. As of

memory, it has been proposed that Orx facilitates spatial learning by exciting hippocampal neurons and by increasing glutamate release in the hippocampus [34]. It has also been shown that Orx-A directly excites local glutamatergic neurons in the PH [35,36]. Thus, Orx may indirectly modulate memory by modulating the glutamate release. It should also be mentioned that other factors including neurotransmitters might change after REMSD that synergistically affect memory formation. Subject to confirmation it may be argued that a non-significant change in Orx-A levels in hippocampus could also be due to opposite change in Orx-A levels in CA1 and CA3 area.

In the present study, we have estimated Orx-A levels in anatomically identified brain regions of different sets of rats maintained under control conditions and those exposed to REMSD. Rats were REMS deprived by the flowerpot method, the most widely used method for such studies [19]. To rule out the non-specific factors confounding the results, we have used FMC, LPC and REC groups. Notwithstanding, like most in vivo studies, arguably there are a few unavoidable technical limitations of using this method, which we have taken care of by using various controls as we have discussed in detail earlier [37,38]. The LPC values were comparable to FMC suggesting that the change in Orx-A level was not due to non-specific factors. Also, as even REMSD associated elevated Orx-A returned to normal FMC level after recovery, it is likely that the effects were specific to REMSD. Further, the effects were not due to any other non-specific factors because the changes in Orx-A level was restricted to some areas only and was not uniform in all brain areas. ELISA method was used for estimation since it is rapid, economical, accurate and also overcomes the limitations of using radioactive methods. Although, micordialysis is a precise method of collecting neurotransmitter from a specific brain area, technically it is quite challenging to surgically implant cannulae/probes in multiple brain regions at the same time in the same rat and collect samples simultaneously from all areas. Further, it is also challenging to maintain such chronically prepaid rats with cannulae for such a long time in REMSD and continue collecting samples at the same time. Thus, given the limitations the information gathered in this study is significant. To our knowledge this is the first study to estimate Orx-A from the different brain regions of the same rat after long-term REMSD and also after recovery.

We conclude that normally Orx-A helps maintaining sleep-waking homeostasis. However, upon REMSD the Orx-A level increases in discrete brain regions causing increased waking and reduced NREMS as well as REMS. The reduced NREMS and REMS could be secondary to increased waking. The effects could be due to activation of glutamatergic or other neurons, which need further studies.

## Conflict of interest

Authors declare no conflict of interest.

## Author's contribution

BNM designed the work; RM and MAK performed experiment and analyzed the data. All authors contributed in writing the final manuscript.

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